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Analysis and Manipulation of Aspartate Pathway Genes for L-Lysine Overproduction from Methanol by *Bacillus methanolicus*▽

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We investigated the regulation and roles of six aspartate pathway genes in L-lysine overproduction in *Bacillus methanolicus*: *dapG*, encoding aspartokinase I (AKI); *lysC*, encoding AKII; *yclM*, encoding AKIII; *asd*, encoding aspartate semialdehyde dehydrogenase; *dapA*, encoding dihydrodipicolinate synthase; and *lysA*, encoding *meso*-diaminopimelate decarboxylase. Analysis of the wild-type strain revealed that *in vivo lysC* transcription was repressed 5-fold by L-lysine and induced 2-fold by DL-methionine added to the growth medium. Surprisingly, *yclM* transcription was repressed 5-fold by DL-methionine, while the *dapG*, *asd*, *dapA*, and *lysA* genes were not significantly repressed by any of the aspartate pathway amino acids. We show that the L-lysine-overproducing classical *B. methanolicus* mutant NOA2#13A52-8A66 has—in addition to a *hom-1* mutation—chromosomal mutations in the *dapG* coding region and in the *lysA* promoter region. No mutations were found in its *dapA*, *lysC*, *asd*, and *yclM* genes. The mutant *dapG* gene product had abolished feedback inhibition by *meso*-diaminopimelate *in vitro*, and the *lysA* mutation was accompanied by an elevated (6-fold) *lysA* transcription level *in vivo*. Moreover, *yclM* transcription was increased 16-fold in mutant strain NOA2#13A52-8A66 compared to the wild-type strain. Overexpression of wild-type and mutant aspartate pathway genes demonstrated that all six genes are important for L-lysine overproduction as tested in shake flasks, and the effects were dependent on the genetic background tested. Coupled overexpression of up to three genes resulted in additive (above 80-fold) increased L-lysine production levels.

The essential amino acid L-lysine is widely used as a feed additive in animal farming, and currently >1,000,000 tons of L-lysine–HCl per year are produced worldwide by fermentation processes, using classical mutant strains of *Corynebacterium glutamicum* and its relatives (1, 7, 34). L-Lysine is a product of the aspartate pathway (Fig. 1), and several of the genes and enzymes involved are feedback regulated. The regulatory mechanisms are allosteric inhibition of the enzymes and transcriptional repression of the genes, including RNA riboswitch mechanisms at the mRNA level (15, 32). The aspartate pathway has been investigated particularly well in *C. glutamicum*, with the overall aim of identifying enzymes representing rate-limiting steps for L-lysine overproduction. In particular, the importance of aspartokinase (AK) in controlling L-lysine biosynthesis has been well documented for this bacterium (13) as well as for several other bacteria, including *Escherichia coli* (25, 30), *Lactobacillus plantarum* (11, 12), *Bacillus subtilis* (41), and *Methylophilus methylotrophus* (38). It has been unraveled that homoserine dehydrogenase (HD), dihydrodipicolinate synthase (DapA), dihydrodipicolinate reductase (DapB), *meso*-diaminopimelate decarboxylase (LysA), and L-lysine exporter (LysE) can also represent targets for achieving L-lysine overproduction (13, 17, 21, 28, 29, 38). Moreover, several enzymes of cell primary metabolism have been found to be important for achieving the efficient precursor supply needed for L-lysine overproduction (22, 40).

Methylotrophic bacteria that can utilize one-carbon compounds such as methanol as their sole carbon and energy source are interesting alternative candidate strains for industrial L-lysine production. Methanol has several unique properties that make it an interesting alternative to molasses for such uses (5, 36). So far, mainly three different methylotrophic bacteria have been explored to various levels for such purposes: the Gram-negative and obligate methylotrophs *Methylophilus methylotrophus* and *Methylobacillus glycogenes* and the Gram-positive, thermotolerant, and restricted methylotroph *Bacillus methanolicus* (5, 7). Several reports on manipulation of the aspartate pathway for L-lysine overproduction exist for both *M. glycogenes* and *M. methylotrophus*. The *M. glycogenes hom* gene, encoding HD, and *thrC* gene, encoding threonine synthase, have been cloned and characterized (27), and overexpression of a mutant *dapA* gene in this bacterium resulted in L-lysine production at 8 g/liter in fermentors (28). Besides these examples, genetic and biochemical information on L-lysine biosynthesis in this bacterium is lacking. The L-lysine biosynthetic pathway of *M. methylotrophus* has been characterized more extensively (16, 38). By manipulating the AK, *dapA*, and *dapB* genes, successive L-lysine overproduction was obtained in this organism. Also, expression of a mutant L-lysine exporter gene, *lysE24*, resulted in increased L-lysine production by this bacterium (17). Disruption of the *metF* gene (encoding 5,10-methylene-tetrahydrofolate reductase), involved in methionine biosynthesis, had a slightly positive effect on lysine synthesis (22). It should be noted that the production levels obtained with recombinant *M. methylotrophus* strains were generally low (up to 1 g/liter in fermentors). To our knowledge, experimental

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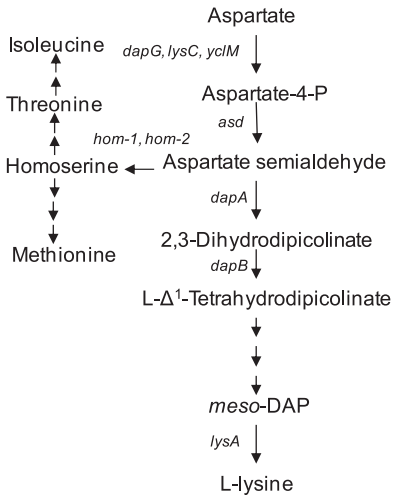

FIG. 1. Overview of the L-aspartate pathway. In total, eight different genes representing the aspartate pathway in *B. methanolicus* have been cloned and sequenced (indicated in the figure), and these genes were investigated here to clarify their regulation of and role in L-lysine overproduction from methanol. *dapG*, AKI gene; *lysC*, AKII gene; *yclM*, AKIII gene; *asd*, aspartate semialdehyde dehydrogenase gene; *hom-1* and *hom-2*, homoserine dehydrogenase I and II genes, respectively; *dapA*, dihydrodipicolinate synthase gene; *dapB*, dihydrodipicolinate reductase gene.

knowledge on feedback repression of aspartate pathway genes in *M. methylotrophus* and *M. glycogenes* is lacking.

Classical mutagenesis approaches have previously been used to isolate amino ethyl cysteine (AEC)-resistant and L-lysine-overproducing *B. methanolicus* mutants, including NOA2#13A52-8A66, recently demonstrated to secrete 47 g/liter of L-lysine under optimized methanol fed-batch fermentations (6). We have shown that *B. methanolicus* has three different AK-encoding genes, namely, *dapG* (encoding AKI), *lysC* (encoding AKII), and *yclM* (encoding AKIII), that are differently feedback inhibited *in vitro*. Interestingly, individual overexpression resulted in up to 60-fold increased L-lysine production (11 g/liter in fed-batch methanol fermentations with *yclM* overexpression) in a wild-type genetic background (24). We later showed that this organism has two different HD-encoding genes, *hom-1* and *hom-2*, and that the classical *hom-1* mutant *B. methanolicus* strain M168-20 had 60-fold increased L-lysine production (6). Interestingly, intracellular metabolite analysis and manipulations with pyruvate carboxylase and citrate synthase indicated that the oxaloacetate precursor supply is not a major bottleneck for L-lysine overproduction in *B. methanolicus* (4, 6).

We present here a broad and systematic analysis aimed at unraveling the roles of six different aspartate pathway genes in L-lysine overproduction in *B. methanolicus*. We included wild-type and classical mutant strains as model strains to analyze transcriptional repression and allosteric regulation and to map beneficial mutations, and we used them as hosts for overexpression of wild-type and mutant genes. Together, our data demonstrate that all six tested genes can have a large impact on L-lysine overproduction in this organism and that multiple aspartate pathway genes should be targeted for manipulations to fully explore the potential of *B. methanolicus* to overproduce L-lysine from methanol.

MATERIALS AND METHODS

Biological materials, DNA manipulations, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were cultivated in Luria-Bertani (LB) broth, and recombinant *E. coli* methods were performed according to standard methods (33). *B. methanolicus* strains were cultivated at 50°C in $MeOH_{200}$ medium containing 200 mM methanol, and transformation of this bacterium was performed by electroporation as described previously (23). Unless otherwise stated, classical *B. methanolicus* mutant strains M168-20 (6) and NOA2#13A52-8A66 were grown in medium supplemented with DL-methionine (1.5 mM), and for the latter strain, L-threonine (1 mM) was also added. For testing of *in vivo* feedback repression by real-time PCR (RT-PCR), cells were cultivated in $MeOH_{200}$ medium supplemented with 10 mM L-lysine, 10 mM L-threonine, and 10 mM DL-methionine as indicated. Recombinant *E. coli* and *B. methanolicus* strains were cultivated in media supplemented with chloramphenicol (15 and 5 μg/ml, respectively). Two different *meso*-diaminopimelate (*meso*-DAP) auxotrophic mutant *E. coli* Hfr3000 strains, U482 and AT997, deficient in aspartate semialdehyde dehydrogenase and dihydrodipicolinate synthase, respectively (10, 20), were used as hosts for complementation with the *B. methanolicus* genes *asd* and *dapA*, respectively. Recombinant *E. coli* Hfr3000 U482 and AT997 strains were tested for growth on LB agar plates, 50 $\mu g\ ml^{-1}$ *meso*-DAP was added when required, and growth was determined after 48 h of incubation at 37°C. Bacterial growth was monitored by measuring the optical density at 600 nm (OD_{600}).

Isolation of total RNA, cDNA synthesis, and RT-PCR. *B. methanolicus* cultures were grown to late logarithmic phase (OD_{600} = 3) before harvesting, and cell lysis, including RNA protection, isolation of total RNA, and concomitant cDNA synthesis, was performed as described elsewhere (23). Total RNA concentrations were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Primers for the RT-PCR experiments were designed by using the computer software Primer ExpressR v 2.0 (Applied Biosystems). Primer sequences are available upon request. Detection of PCR products was performed with an ABI 7500 system (Applied Biosystems), essentially as described previously (6). Data acquisition and analysis were performed with Sequence Detection software, version v1.2.3 (Applied Biosystems Inc.), under standardized reaction conditions with a Sybr signal.

Construction of expression vectors. (i) pTH1mp-dapG, pTH1mp-dapG_D375E, pTH1mp-asd, pTH1mp-dapA, and pTH1mp-lysA. DNA fragments with the coding regions of *dapG* (1,260 bp), *asd* (1,117 bp), *dapA* (904 bp), and *lysA* (1,322 bp) were PCR amplified from *B. methanolicu*s MGA3 total DNA, and a DNA fragment with the coding region of *dapG*(*D375E*) (1,260 bp) was PCR amplified from *B. methanolicus* mutant NOA2#13A52-8A66 total DNA, using the following primer pairs: dapG-F, 5′-TTTTACATGTGAAAATTATCGTTCAAAAATTCGGG-3′; dapG-R, 5′-TTTTGGTACCTCATTCTATCCGTTCAAACTCC-3′; asd-F, 5′-GCGCACATGTGGGTCAAGAAAATGGTCTTC-3′; asd-R, 5′-ATGGTACCTGCCCCCGAATTTTTGAAC-3′; dapA-F, 5′-GCGCACATGTGGTTTCATTTGGTCGAATATC-3′; dapA-R, 5′-ATGGTACCGGCAGTAAAAACTCCATTGAT-3′; lysA-F, 5′-GCGCACATGTGTATTTTCATGGCACAACA-3′; and lysA-R, 5′-ATGGTACCGCAGCTTAGTATCTTACTCT-3′.

PciI and KpnI restriction sites are underlined in the forward and reverse primers, respectively. The obtained PCR products were end digested with PciI and KpnI and ligated into the corresponding sites of pTH1mp-lysC (replacing the *lysC* gene), yielding the expression vectors pTH1mp-dapG, pTH1mp-dapG_D375E, pTH1mp-asd, pTH1mp-dapA, and pTH1mp-lysA.

(ii) pTH1mp-dapA-yclM. Plasmid pHP13mp-yclM was digested with SpeI and NcoI, and the 1,902-bp *yclM* fragment was cloned into the compatible XbaI and NcoI sites of plasmid pTH1mp-dapA.

(iii) pTH1mp-dapA-yclM-lysA. Plasmid pTH1mp-lysA was digested with SpeI and NcoI, and the 1,834-bp *lysA* fragment was cloned into the compatible XbaI and NcoI sites of pTH1mp-dapA-yclM.

All constructed plasmids were verified by DNA sequencing.

Analysis of L-lysine production. In order to test a large number of different strains, L-lysine production experiments were performed in shake flasks as described previously (6). Frozen ampoules prepared from exponentially growing *B. methanolicus* cells were cultivated at 50°C in 100 ml $MeOH_{200}$ medium in baffled 500-ml shake flasks until the exponential growth phase (OD_{600} = 1 to 2). From these cultures, cells were diluted 1:20 in fresh prewarmed medium, and cell growth was continued. At least three samples for amino acid measurements were collected during the late exponential and stationary growth phases, and measurements of L-lysine were performed by using high-performance liquid chroma-

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype[a]	Reference or source
Strains		
E. coli strains		
XL1 Blue	General cloning host	Stratagene
Hfr3000 U482	*asd* negative; *meso*-DAP auxotrophic mutant	20
AT997	*dapA* negative; *meso*-DAP auxotrophic mutant	10
ER2566	Expression host, carries chromosomal gene for T7 RNA polymerase	New England Biolabs
B. methanolicus strains		
MGA3	Wild-type strain ATCC 53907	35
M168-20	AEC-resistant MGA3 mutant	6
NOA2#13A52-8A66	AEC-resistant NOA2 mutant	19
Plasmids		
pHP13	*B. methanolicus-E. coli* shuttle vector; Clm^r	18
pSB1	pET21a with *dapG* coding region under control of the T7 promoter and fused to six-His tag	24
pSB5	Similar to pSB1, with *dapG* replaced with *dapG*(*D375E*) coding region	This study
pTH1mp-lysC	Similar to pHP13mp-lysC, but with PciI site upstream of the *mdh* promoter removed	6
pTH1mp-dapG	pTH1 with *dapG* coding region under control of the *mdh* promoter	This study
pTH1mp-dapG_D375E	pTH1 with *dapG*(*D375E*) coding region of mutant strain NOA2#13A52-8A66 under control of the *mdh* promoter	This study
pTH1mp-asd	pTH1mp-lysC with *lysC* coding region replaced with *asd* coding region	This study
pTH1mp-dapA	pTH1mp-lysC with *lysC* coding region replaced with *dapA* coding region	This study
pTH1mp-lysA	pTH1mp-lysC with *lysC* coding region replaced with *lysA* coding region	This study
pTH1mp-dapA-yclM	pTH1mp-dapA with *yclM* coding region downstream of *dapA*	This study
pTH1mp-dapA-yclM-lysA	pTH1mp-dapA-yclM with *lysA* coding region downstream of *yclM*	This study

[a] Clm^r, chloramphenicol resistance. The construction of plasmids made in this study is described further in the text.

tography (HPLC) (6, 24). Maximum L-lysine production yields were typically obtained 3 to 5 h after cell cultures entered the stationary phase and remained high for up to 24 h.

Purification and biochemical characterization of *dapG*(*D375E*) gene product (AKI-D375E). The mutant *dapG* gene of NOA2#13A52-8A66, designated *dapG*(*D375E*), was used to substitute for the *dapG* coding region in plasmid pSB1 (24), and the resulting plasmid, denoted pSB5 (Table 1), was transformed into *E. coli* ER2566. The resulting strain, *E. coli* ER2566(pSB5), and its parental strain, *E. coli* ER2566(pSB1), were used for recombinant production and concomitant purification of AKI-D375E and wild-type AKI, respectively. Typically, two independent protein purifications were performed. The purified proteins were subjected to *in vitro* biochemical characterization, including determination of kinetic (aspartate K_m [$K_{m\ ASP}$] and V_{max}) and inhibition (50% inhibitory concentration [IC_{50}]) constants, as described previously (24).

RESULTS AND DISCUSSION

The *B. methanolicus asd* and *dapA* genes encode active aspartate semialdehyde dehydrogenase and dihydrodipicolinate synthase, respectively. The biochemical functions of the *B. methanolicus lysA* (26) and *dapG*, *lysC*, and *yclM* (24) gene products have been verified experimentally. We recently cloned the *asd* and *dapA* genes (24), but the biological functions of the respective gene products were not confirmed experimentally. Therefore, plasmids pTH1mp-dapA and pTH1mp-asd (Table 1), expressing *dapA* and *asd*, respectively, from the strong *mdh* promoter, were constructed. The plasmids were transformed into the *meso*-DAP auxotrophic *E. coli* mutants Hfr3000 U482 and AT997 (Table 1), deficient in *asd* and *dapA*, respectively (Table 1), for phenotypic complementation experiments. We showed that recombinant strains Hfr3000 U482(pTH1mp-asd) and AT997(pTH1mp-dapA) both grew well in defined minimal medium without supplementation of *meso*-DAP, while the mutant host strains carrying the empty vectors were not able to grow under such conditions (data not shown). These results confirmed that the *B. methanolicus asd* and *dapA* genes complemented aspartate semialdehyde dehydrogenase and dihydrodipicolinate synthase mutations, respectively, in *E. coli*, in agreement with the predicted functions for these two genes.

Transcription of *lysC* and *yclM* is repressed by L-lysine and DL-methionine, respectively, in wild-type *B. methanolicus* MGA3. We recently demonstrated that *B. methanolicus* has two HD-encoding genes, *hom-1* and *hom-2*, and that they are repressed transcriptionally by L-threonine and DL-methionine, respectively (6). To investigate the occurrence of analogous feedback repression of the six relevant aspartate pathway genes, wild-type *B. methanolicus* MGA3 was cultivated in the presence of 10 mM L-lysine, L-threonine, DL-methionine, or L-lysine plus L-threonine until late logarithmic phase, before cell harvesting and analysis by RT-PCR. The growth rates of the cell cultures were essentially similar under these different conditions (data not shown). The results of these experiments (Table 2) showed that the *lysC* transcription level was repressed 5-fold by the added L-lysine and induced >2-fold by DL-methionine. Surprisingly, transcription of *yclM* was repressed 5-fold by DL-methionine, while L-lysine and L-threonine either alone or together had minor effects on the transcription level of this gene. This is different from the case in *B. subtilis*, where L-lysine and L-threonine act as corepressors for both *lysC* and *yclM* transcription, and in this organism, *yclM* transcription is induced by L-lysine (3). In *C. glutamicum*, L-methionine represses homoserine dehydrogenase activity, leading to reduced L-threonine synthesis and increased L-lysine secretion (39). The MGA3 *asd*, *dapA*,

TABLE 2. Transcriptional repression of L-aspartate pathway genes in wild-type *B. methanolicus* MGA3[a]

Gene	Relative transcription level in the presence of amino acid(s)				
	None	L-Lysine	L-Threonine	DL-Methionine	L-Lysine + L-threonine
dapG	1.0 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	0.8 ± 0.2	0.9 ± 0.1
lysC	1.0 ± 0.2	0.2 ± 0.05	0.6 ± 0.1	2.1 ± 0.2	0.3 ± 0.05
yclM	1.0 ± 0.2	1.2 ± 0.3	0.7 ± 0.2	0.2 ± 0.05	0.9 ± 0.2
asd	1.0 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	0.8 ± 0.2	1.1 ± 0.2
dapA	1.0 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	0.9 ± 0.2
lysA	1.0 ± 0.2	0.8 ± 0.2	0.9 ± 0.2	1.2 ± 0.2	0.9 ± 0.3
hom-1[b]	1.0	1.2	0.03	0.6	NT
hom-2[b]	1.0	1.0	0.43	0.07	NT

[a] Wild-type *B. methanolicus* cells were supplemented with 10 mM amino acids and cultivated for another 1 h before cell harvesting and RT-PCR experiments (see Materials and Methods). At least three parallel experiments were performed, and the mean values with standard errors are given. NT, not tested.
[b] Data for *hom-1* and *hom-2* are from reference 6.

and *dapG* genes are members of the *dap* operon (24), and transcription levels for these three genes were not significantly affected (<2-fold) by any of these amino acids, similar to results reported for *B. subtilis* (3). The same conclusion was also obtained for the *lysA* gene, while the corresponding gene in *B. subtilis* is repressed by L-lysine (3). Together, these data demonstrate several differences in regulation of aspartate pathway genes in *B. methanolicus* and *B. subtilis*.

L-Lysine-overproducing *B. methanolicus* mutant NOA2# 13A52-8A66 has chromosomal mutations in the *hom-1*, *dapG*, and *lysA* genes. We next took advantage of the AEC-resistant *B. methanolicus* mutant strain NOA2#13A52-8A66 (19) as a model strain to map mutations beneficial for L-lysine overproduction. This mutant can overproduce L-lysine (47 g/liter) in optimized fed-batch methanol fermentations, and we recently determined that this strain has a chromosomal *hom-1* mutation (6). Total DNA was isolated and used as a template for PCR-assisted amplification and concomitant DNA sequencing of the *dapG*, *lysC*, *yclM*, *asd*, *dapA*, and *lysA* genes, including at least 270-bp upstream regions to also identify any mutations in the promoter and untranslated leader regions. The results of these experiments are summarized in Table 3. The NOA2#13A52-8A66 mutant had one point mutation in the *dapG* coding region (C at position +1125 replaced with A, leading to the amino acid substitution D375E in the corresponding gene product) and one point mutation in the *lysA* upstream region (T at position −70 mutated to A). The remaining genes tested had no mutations. As controls, we included the classical *hom-1* mutant strain M168-20 (6) and wild-type strain MGA3 in similar analyses. As expected, no chromosomal mutations were found in any of the six genes for these two strains. In summary, these data indicate that *dapG* and *lysA*, together with the previously documented *hom-1* gene, represent key mutational targets for AEC resistance and L-lysine overproduction by *B. methanolicus*.

Transcription levels for the *yclM* and *lysA* genes are elevated 16-fold and 6-fold, respectively, in the NOA2#13A52-8A66 mutant compared to wild-type strain MGA3. Regulation of individual genes can be affected by polar mutations in distantly located genes (9), and for inverse metabolic engineering, a complementary strategy to mutational mapping is to look for alterations in transcriptional regulation in relevant mutant strains (2, 8). For example, we previously showed that *pyc* transcription was upregulated 3-fold in the NOA2#13A52-8A66 mutant compared to the wild type, but no mutations were found in the *pyc* locus of this mutant strain (6). We therefore proceeded to analyze transcription levels for the aspartate pathway genes in this mutant by using RT-PCR as described above. To obtain comparable conditions for growth, cells were cultivated in methanol medium supplemented with 1 mM DL-methionine and 1.5 mM L-threonine (see Materials and Methods), as required for growth of NOA2#13A52-8A66 (19). Both MGA3 and M168-20 were included as control strains, and the results of these experiments are shown in Table 4. Interestingly, the two mutant strains both displayed significantly upregulated *yclM* transcription compared to the wild-type strain (12- and 16-fold, respectively). A plausible expla-

TABLE 3. Mutational mapping in *B. methanolicus* mutant strains

B. methanolicus mutant	Presence of mutation in gene[a]		
	dapG	*lysA*	*hom-1*[b]
M168-20	No	No	Yes[c]
NOA2#13A52-8A66	Yes[d]	Yes[e]	Yes[f]

[a] At least 270 bp upstream of the coding region was included in the DNA sequence analysis for all genes. No mutations were seen in the *lysC*, *yclM*, *asd*, *dapA*, and *hom-2* genes.
[b] Data for the *hom-1* and *hom-2* genes are from reference 6.
[c] *hom-1* substitution mutations G → A (D120N) and G → T (V397F), at positions +357 and +1188, respectively.
[d] *dapG* substitution mutation C → A at position +1125 (D375E).
[e] *lysA* promoter mutation T → A at position −70.
[f] *hom-1* substitution mutation G → T at position +784 (V262F).

TABLE 4. Transcriptional regulation of L-aspartate pathway genes in L-lysine-overproducing classical *B. methanolicus* mutant strains[a]

Gene	Relative transcription level in *B. methanolicus* strain			
	MGA3	MGA3 (−MT)	M168-20	NOA2#13A52-8A66
asd	1.0 ± 0.2	0.9 ± 0.3	1.2 ± 0.1	1.3 ± 0.3
dapA	1.0 ± 0.2	1.2 ± 0.2	1.4 ± 0.2	1.5 ± 0.3
dapG	1.0 ± 0.2	1.1 ± 0.2	1.8 ± 0.1	1.6 ± 0.3
lysA	1.0 ± 0.1	1.0 ± 0.2	1.4 ± 0.3	6.0 ± 1.0
lysC	1.0 ± 0.1	0.7 ± 0.2	0.4 ± 0.1	0.4 ± 0.05
yclM	1.0 ± 0.2	2.0 ± 0.4	12.0 ± 2.0	16 ± 2.5

[a] Exponentially growing cells were harvested and analyzed by RT-PCR (see Materials and Methods). −MT, DL-methionine and L-threonine were not added. Mutant strains M168-20 and NOA2#13A52-8A66 were not tested under "−MT" conditions due to poor growth in the absence of these amino acids (see Materials and Methods). Data are means with standard errors.

TABLE 5. Biochemical properties of the *dapG*(*D375E*) gene product, AKI-D375E, compared to the wild-type *dapG* gene product, AKI[a]

Enzyme	$K_{m\ ASP}$ (mM)	V_{max} (μmol/min/mg protein)	*meso*-DAP IC_{50} (mM)
AKI	5.0	47	0.1
AKI-D375E	3.5	45	>20

[a] Kinetic ($K_{m\ ASP}$ and V_{max}) and inhibition (IC_{50}) constants were calculated as described previously (24). The purified proteins were tested for catalytic activity in the presence of *meso*-DAP concentrations of up to 20 mM, and AKI-D375E was not inhibited under any of the conditions tested. The results from multiple independent experiments were essentially similar, and representative values are given.

TABLE 6. L-Lysine production yields obtained in shake flask cultures of recombinant *B. methanolicus* strains[a]

Plasmid	Overexpressed gene(s)	Host strain[b]	L-Lysine concn (mg/liter)[c]
pHP13	None	MGA3	7 ± 1
pHP13mp-dapG	*dapG*	MGA3	7 ± 1
pHP13mp-lysC	*lysC*	MGA3	55 ± 5
pHP13mp-yclM	*yclM*	MGA3	140 ± 10
pTH1mp-lysA	*lysA*	MGA3	150 ± 10
pTH1mp-dapA	*dapA*	MGA3	7 ± 1
pTH1mp-asd	*asd*	MGA3	7 ± 1
pTH1mp-dapG_D375E	*dapG*(*D375E*)	MGA3	120 ± 10
pTH1mp-dapA-yclM	*dapA* + *yclM*	MGA3	210 ± 20
pTH1mp-dapA-yclM-lysA	*dapA* + *yclM* + *lysA*	MGA3	580 ± 30
pHP13	None	M168-20	150 ± 10
pTH1mp-asd	*asd*	M168-20	280 ± 20
pTH1mp-dapA	*dapA*	M168-20	700 ± 40[d]
pTH1mp-dapA-yclM	*dapA* + *yclM*	M168-20	660 ± 40

[a] Recombinant strains were grown in methanol medium in shake flasks for amino acid production measurements as described previously (6). All experiments were run in triplicate, and the mean values with standard errors are given.
[b] DL-Methionine was added when M168-20 was used as the host.
[c] In all cases, maximum L-lysine levels are presented (see Materials and Methods).
[d] L-Threonine-supplemented growth medium was used due to poor cell growth (see Materials and Methods).

nation might be that the *hom-1* mutation causes low L-methionine levels and concomitant upregulation of *yclM* transcription in these mutant cells. Taken together with our previous data showing that *yclM* overexpression alone can cause about 60-fold increased L-lysine production in MGA3 (24), it is plausible to assume that this elevated *yclM* expression is one major contribution to the elevated L-lysine production levels of both M168-20 and NOA2#13A52-8A66. For the NOA2#13A52-8A66 mutant, the *lysA* transcription level was also upregulated 6-fold, possibly mediated by the identified point mutation in the *lysA* upstream region (Table 3). We also noticed that the *lysC* transcript level was repressed 2.5-fold in both mutants compared to the wild-type strain. Possibly, *lysC* transcription was repressed by the increased L-lysine production levels in these two classical mutant strains. The transcriptional regulation of the remaining three *dap* operon genes tested, *asd*, *dapG*, and *dapA*, was not significantly affected (<2-fold) in either of the two mutant strains.

To rule out whether the DL-methionine and L-threonine added to the growth medium in these experiments may have directly caused any feedback repression of the genes tested (Table 2), MGA3 was also analyzed upon growth without these two amino acids supplied. The results from these experiments demonstrated that *yclM* transcription was about 2-fold higher under these conditions, while the remaining genes tested were transcribed at essentially similar levels under both conditions tested (Table 4). In summary, these results confirm that L-lysine overproduction by the NOA2#13A52-8A66 mutant is accompanied by elevated transcription levels for *yclM* and *lysA* and a reduced transcription level for *lysC*.

The *dapG*(*D375E*) gene product AKI-D375E has abolished *meso*-DAP inhibition *in vitro*. The *dapG*(*D375E*) gene was expressed as a recombinant His_6-tagged fusion protein in *E. coli* by use of plasmid pSB5 (Table 1) and was purified by affinity chromatography. Purified enzymes were subjected to *in vitro* biochemical characterizations as described previously (24), and the results demonstrated that the $K_{m\ ASP}$ and V_{max} values of AKI-D375E remained similar to those of the wild-type AKI enzyme (Table 5). Interestingly, *meso*-DAP concentrations of up to 20 mM had no detectable effect on AKI-D375E catalytic activity (Table 5). This was in contrast to the case for the wild-type AKI protein, which has an inhibition constant (IC_{50}) of 0.1 mM for this compound (24), and confirmed that this mutant enzyme has abolished allosteric inhibition by *meso*-DAP. It was plausible to assume that this deregulation contributed to the L-lysine overproduction of the NOA2#13A52-8A66 mutant, and this is confirmed below.

Individual overexpression of aspartate pathway genes increases L-lysine production up to 20-fold in a wild-type *B. methanolicus* genetic background. We previously constructed the recombinant strains MGA3(pHP13mp-dapG), MGA3(pHP13mp-lysC), and MGA3(pHP13mp-yclM) and demonstrated that they displayed 2-, 10-, and 60-fold elevated L-lysine production yields compared to the control strain MGA3(pHP13) in fed-batch methanol fermentations (24). These three strains were included as control strains, together with the NOA2#13A52-8A66 mutant, in the present production analyses (see below). Plasmid pHP13mp-lysC was later modified into the cassette cloning and expression vector pTH1mp-lysC (6), and the *asd*, *dapA*, *lysA*, and *dapG*(*D375E*) coding regions were used to substitute for *lysC* in this vector. All constructed plasmids were transformed into MGA3, and all recombinant strains were tested for L-lysine production in shake flask methanol cultivations (Table 6). Strain MGA3(pTH1mp-dapG) produced L-lysine at wild-type levels (7 mg/liter), while MGA3(pHP13mp-lysC) and MGA3(pHP13mp-yclM) displayed 8-fold and 20-fold (up to 140 mg/liter) increased L-lysine production levels, respectively, compared to control strain MGA3 (pHP13) under these conditions. As a control, we included the classical mutant NOA2#13A52-8A66(pHP13), which secreted 900 mg/liter of L-lysine under these conditions. Together, these data are in general good agreement with the analogous fermentation data for these three recombinant strains (6, 24), indicating that the relative L-lysine production levels obtained under the present conditions reflect the analogous data obtained in fed-batch fermentations.

Recombinant strain MGA3(pTH1mp-lysA) displayed a 20-fold increased L-lysine production level compared to that of MGA3(pHP13), while MGA3(pTH1mp-dapA) and MGA3(pTH1mp-asd) both produced L-lysine at wild-type lev-

els (Table 6). Both the *lysC* and *lysA* gene products are inhibited by L-lysine (24, 26), demonstrating that overexpression of feedback-inhibited enzymes of the aspartate pathway can have surprisingly good effects on L-lysine production yields in a wild-type *B. methanolicus* host. In contrast, while *dapG* overexpression had no effect on L-lysine production, recombinant strain MGA3(pTH1mp-dapG_D375E) secreted 120 mg/liter of L-lysine under such conditions. The latter results are in good agreement with the different biochemical data for the AKI-D375E protein and the native AKI protein (see above), and together our data imply that all three AKs can play important roles in L-lysine overproduction in *B. methanolicus* strains. In summary, in addition to the previously documented important roles of *lysC* and *yclM* (24), these data imply that *lysA* and *dapG* can also play key roles in L-lysine overproduction by *B. methanolicus*. In contrast, overexpression of the *asd* and *dapA* genes had no impact on L-lysine overproduction under these conditions.

Overexpression of *asd* and *dapA* results in 1.8-fold and 4.4-fold (up to 700 mg/liter) increased L-lysine production levels in the M168-20 genetic background. For *C. glutamicum*, it has been demonstrated that the effect of manipulating single genes involved in L-lysine overproduction may strongly depend on the genetic background tested (31). We therefore decided to use the classical *hom-1* mutant, L-lysine-overproducing strain M168-20 as an alternative host to further investigate the effect of overexpressing selected aspartate pathway genes. To investigate the roles of *dapA* and *asd*, in particular, in L-lysine production in *B. methanolicus*, the recombinant strains M168-20(pTH1mp-dapA) and M168-20(pTH1mp-asd) were established and tested for L-lysine production as described above (Table 6). L-Lysine production by M168-20(pTH1mp-asd) was increased about 1.9-fold compared to that of control strain M168-20(pHP13). M168-20(pTH1mp-dapA), on the other hand, displayed poor growth under these conditions (data not shown), even though DL-methionine was added to the growth medium. Similar growth effects have been reported for analogous experiments using L-lysine-overproducing *C. glutamicum* hosts (14). We hypothesized that DapA overexpression may drain carbon away from the biosynthetic branch, leading to synthesis of L-methionine and L-threonine in the cells (Fig. 1). We therefore repeated this experiment and supplied L-threonine in addition to DL-methionine to the growth medium (see Materials and Methods). Under these conditions, the growth rate was improved but was still slightly lower than that of the control strain M168-20(pHP13) (data not shown), confirming an L-threonine shortage in these recombinant cells. In any case, L-lysine production by this recombinant strain was improved 4.7-fold (700 mg/liter) compared to that by the control strain M168-20(pHP13). In summary, these results clearly demonstrate that both *asd* and *dapA* may be key genes for achieving L-lysine overproduction by *B. methanolicus* and that the effects are highly dependent on the genetic background tested.

Overexpression of multiple L-aspartate pathway genes causes additive increments in L-lysine production levels (up to 580 mg/liter) in the wild-type MGA3 genetic background. The accumulated results obtained clearly indicated that all six tested aspartate pathway genes can play key roles in regulating the L-lysine production level in *B. methanolicus*. Motivated by these findings, we proceeded to coexpress alternative combinations of multiple genes in the wild-type MGA3 genetic background to test if L-lysine production could be improved further. The vectors were designed such that all recombinant genes were cotranscribed from the strong *mdh* promoter, and each recombinant gene was similarly engineered in frame with the *mdh* ribosome binding site to also ensure efficient translation (6). The recombinant strain MGA3(pTH1mp-dapA-yclM) displayed about 1.5-fold more L-lysine production than strain MGA3(pHP13mp-yclM) under these conditions (Table 6). To test if the marginal improvement obtained by coexpressing *dapA* and *yclM* versus expressing *yclM* alone may have been due to a high flux of carbon toward the branch catalyzed by HD (Fig. 1), we repeated this experiment in the *hom-1* mutant strain M168-20 background. Recombinant strain M168-20(pTH1mp-dapA-yclM) produced 660 mg/liter of L-lysine (Table 6), and in contrast to the mother strain, M168-20(pTH1mp-dapA), it grew well without any extra L-threonine supplied to the growth medium. This result implies that coexpression of *dapA* together with *yclM* leads to increased aspartate semialdehyde levels, providing sufficient biosynthesis of threonine for bacterial growth.

Encouraged by the positive results of coexpressing *dapA* and *yclM*, we next tested if combinatorial overexpression of the *lysA* gene, representing the final step in the L-lysine biosynthetic pathway, could also lead to further improved L-lysine overproduction in the wild-type MGA3 genetic background. Thus, vector pTH1mp-dapA-yclM-lysA was constructed and transformed into MGA3, and the resulting recombinant strain displayed almost 3-fold increased L-lysine production (580 mg/liter) compared to the parental strain, MGA3(pHP13mp-dapA-yclM) (Table 6). Thus, coexpression of LysA contributed to improved carbon flow toward L-lysine production. We noticed that MGA3(pTH1mp-dapA-yclM-lysA) had a reduced growth rate under these conditions, and this could not be compensated for by adding L-threonine and DL-methionine (data not shown). The biological reason for this reduction in growth is unknown.

In conclusion, these results clearly demonstrate that coupled overexpression of up to three genes can confer additive positive effects on L-lysine production levels in *B. methanolicus*, and by using this strategy, we obtained a >80-fold increased L-lysine production level (580 mg/liter) in the wild-type genetic background under the conditions tested. Our results indicate that multiple aspartate pathway genes can play important roles in L-lysine overproduction by *B. methanolicus*, and we could not rule out the possibility that genes outside this pathway are important as well. For example, microarray data have revealed a number of novel genes outside the aspartate pathway with important roles in L-lysine overproduction by the classical *C. glutamicum* mutant MH20-22B, including a putative methyltransferase gene and the *amtA-ocd-soxA* operon, encoding an ammonium uptake system (37). Further investigations will determine if similar findings will be observed for *B. methanolicus*.

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